APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1995, p. 583–591 Vol. 61, No. 2
0099-2240/95/$04.00+0

Ruminal Microbial Digestion in Free-Living, in Captive Lichen-Fed, and in Starved Reindeer (*Rangifer tarandus tarandus*) in Winter

TOVE H. AAGNES,* WENCHE SØRMO, AND SVEIN D. MATHIESEN

Department of Arctic Biology and Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway

Received 25 July 1994/Accepted 30 November 1994

In free-living (FL) reindeer eating a natural mixed winter diet dominated by lichens, captive (CF) reindeer fed pure lichens ad libitum, and CF reindeer subsequently starved for 1 day (CS1 reindeer) or 4 days (CS4 reindeer), the dominant rumen anaerobic bacteria were characterized, their population densities were estimated, and ruminal pH and volatile fatty acid concentrations were determined. In the FL reindeer, the total median viable anaerobic bacterial population ranged from 18×10^8 to 35×10^8 cells per ml of rumen fluid ($n = 4$), compared with 26×10^8 to 34×10^8 and 0.09×10^8 to 0.1×10^8 cells per ml of rumen fluid in CF reindeer ($n = 2$) and CS4 reindeer ($n = 2$), respectively. The median bacterial population adhering to the rumen solids ranged from 260×10^8 to 450×10^8, 21×10^8 to 38×10^8, and 0.5×10^8 to 1.9×10^8 cells per g (wet weight) of rumen solids in FL, CF, and CS4 reindeer, respectively. Although there were variations in the rumen bacterial composition among the FL reindeer ($n = 4$), strains of *Bacteroides*, *Fibrobacter*, *Streptococcus*, and *Clostridium* dominated in the rumen fluid. *Streptococcus* spp. and *Clostridium* spp. were the dominant bacteria in the CF reindeer ($n = 2$), while in the CS4 reindeer ($n = 2$) the dominant bacteria were *Fusobacterium* spp., members of the family *Enterobacteriaceae*, and *Eubacterium* spp. Transmission electron micrographs of lichen particles from the rumen of one FL reindeer, one CF reindeer, and one CS4 reindeer show bacteria resembling *Bacteroides* spp. adhering to the lichen particles, evidently digesting the lichen hyphae from the inside. The median ruminal volatile fatty acid concentrations and acetate/propionate ratios were 78.9 mmol/liter and 4.0, respectively, in the FL reindeer ($n = 4$), compared with 66.7 mmol/liter and 3.0 in the CF reindeer ($n = 4$) and 19.9 mmol/liter and 5.3 in the CS4 reindeer ($n = 4$). In comparison with a pure lichen diet, a mixed natural winter diet seems to increase the bacterial numbers associated with the rumen solid fraction and to increase rumen fermentation in favor of plant fiber digestion. Starvation greatly reduced the bacterial population densities and changed the bacterial species composition in the rumen.

Lichens, composed of fungi and algae, are chemically and structurally very different from vascular plants. The cell walls in grasses consist mainly of cellulose (34 to 68%), hemicellulose (34 to 60%), and lignin (5 to 17%) (39). The hemicelluloses include xylans (in β-1,4 linkage), and xyloglucans are also present (39). Lichen cell walls consist mainly of hemicellulose and the lichen starch lichenin (in β-1,4 and β-1,3 linkages) (8, 13). Wild reindeer in southern Norway eat mainly *Cladonia* lichens during the winter (35). One of these, *Cladonia stellaris*, contains 3.1% of its dry matter (DM) as crude protein (20) and 78.4% as hemicellulose but only 1.7% as cellulose (34) and 2.0% as water-soluble carbohydrates (12). Free-living (FL) reindeer in northern Norway are semidomesticated, and the herds migrate between distinct summer and winter pastures because of pronounced seasonal changes in both the quality and availability of food. In summer, reindeer select vascular plants of high quality. Recently, Mathiesen et al. (26) found that FL reindeer in northern Norway in winter eat a mixed diet dominated by lichens. The principal types of plants in the rumens of these animals were lichens (36.2%), woody plants (28.4%), grasses (12.3%), and mosses (6.7%), and the chemical composition of the DM of the plant fraction of the rumen content was 9.6% crude protein, 46.3% hemicellulose, 19.2% cellulose, and 12.4% lignin. The proportion of a pure lichen diet that is digestible by reindeer is 75% (20), which indicates a high level of fermentation of the lichens in the rumen. Little is known about the rumen microorganisms in reindeer that feed on lichens in Norway. However, in an Alaskan reindeer that ate a natural mixed diet including lichens and thereafter was fed a ration of dry lichens for 14 days, 13 dominant strains of rumen bacteria of genera such as *Butyrivibrio*, *Treponema*, *Streptococcus*, and *Lactobacillus* were found (9).

In winter (November to May), most of the reindeer range is covered by snow, and the Gulf Stream along the coast of northern Norway contributes to an unstable winter climate. Temperatures periodically rise above freezing even in midwinter, and such periods followed by periods of subzero temperatures may produce a crust of ice on the snow through which the reindeer cannot dig to reach the plants beneath. Such conditions expose the reindeer to periods of acute starvation. The rumen normally supports a mixed microbial population specially adapted to the fermentation of plant tissue (17). The influence of starvation on the species composition of bacteria in the reindeer rumen is not known, although Mathiesen et al. (27) showed that the mean population densities of rumen bacteria and ciliates decreased during 3 days of starvation, a phenomenon which could contribute to digestion disorders when food is available again. In view of the unusual nature of lichens, the bacterial characteristics and fermentation patterns in the rumens of captive (CF) reindeer fed pure lichens were compared with those of (FL) reindeer eating a mixed diet dominated by lichens on a natural winter pasture. Changes in the rumen bacterial characteristics and fermentation pattern during 4 days of starvation were examined for the captive reindeer that were previously fed lichens (CS1 reindeer [starved for 1 day] and CS4 reindeer [starved for 4 days]).

* Corresponding author. Phone: 47 77 64 47 90. Fax: 47 77 64 57 70. Electronic mail address: toveaa@fagmed.uit.no.

MATERIALS AND METHODS

Animals. Four adult female FL reindeer from a herd in northern Norway (69°N, 23°E) were killed in February 1991 on a natural Arctic alpine pasture where they had been eating a mixed diet dominated by lichens. The animals were killed by decerebration and bleeding by skilled reindeer herders. After death, the animals were transported to a field laboratory, where measurements commenced within 30 min. The gastrointestinal tract was removed from each animal for collection of rumen samples.

Four adult CF reindeer fed pure lichens ad libitum and subsequently starved for 1 day (CS1 reindeer) and for 4 days (CS4 reindeer) are described in a previous paper (1). These animals were equipped with permanent rumen cannulas (11, 21) and familiarized with frequent handling. They had previously been given a commercial reindeer feed (RF-80) (3) and were therefore each inoculated in February 1989 with 500 ml of fresh rumen fluid obtained from an FL reindeer eating a natural mixed winter diet. Thereafter, the animals were fed a pure lichen diet ad libitum for 5 weeks before collection of rumen samples. This diet consisted mostly of *Cladonia stellaris* and also include *Cetraria nivalis*, *Cladonia arbuscula*, and *Cladonia rangiferina*. After 7 weeks on the lichen diet, the animals were starved for 4 days and then fed lichens again. During the feeding period, the animals were kept in individual snow-covered paddocks connected to pens with slatted floors. In the starvation period, the animals were kept in the pens. Snow and salt blocks were available for the animals at all times.

Enumeration of bacteria. Samples of rumen contents were collected from the four FL reindeer within 40 min after death. Samples were collected from two of the CF, CS1, and CS4 reindeer, by aspiration through the rumen fistula from five widely separated parts of the rumen, bulked, and then subsampled. These samples were taken in the morning just before feeding and at the same time of day during the first and the fourth days of starvation. Rumen fluid was obtained by straining rumen contents through two layers of muslin. Colony counts of viable cells present in dilutions of rumen fluid were made by the techniques of Hungate (16) and Orpin et al. (32) with an anaerobic rumen culture medium (M8L medium) modified from those described by Bryant and Robinson (4) and Dehority and Grubb (10). M8L medium contained a basal medium (M8 medium) supplemented with glucose, cellobiose, *N*-acetyl-D-glucosamine, mannose, starch, and lichenin, each at a concentration of 0.2% (wt/vol). The lichenin used (catalog no. L 6133; Sigma) was from the lichen *Cetraria islandica*. The medium was solidified with 2.0% (wt/vol) agar. When rumen bacteria from the CF, CS1, and CS4 reindeer were counted, 0.2% (wt/vol) concentrations of fructose and galactose were added to the medium. The contents of M8 medium and the procedure for making a rumen culture medium are described by Olsen et al. (31). The rumen fluid was diluted serially in 10-fold steps in M8 medium in Hungate anaerobic culture tubes (catalog no. 2047/16-125; Bellco, Vineland, N.J.). For the FL reindeer, the numbers of viable cells of bacteria present in dilutions of 10^{-6} to 10^{-9} were estimated by plating the dilutions in quadruplicate in petri dishes containing M8L medium in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.) in an atmosphere of N_2, CO_2, and H_2. A palladium catalyst was present to remove O_2, and the temperature in the chamber was 39°C. The bacteria were grown under a CO_2 atmosphere in sealed boxes in the chamber. For the CF, CS1, and CS4 reindeer, the bacterial cells present in dilutions of 10^{-5} to 10^{-8} were inoculated in quadruplicate into Hungate culture tubes containing M8L medium. Bacterial colonies on the petri dishes and in the culture tubes were counted after 48 h of incubation at 39°C. The numbers of bacteria adhering to the rumen solids were estimated by a modified version of the technique used for estimating numbers of bacteria in the rumen fluid. Rumen solids obtained by straining the rumen contents through two layers of muslin were suspended for 30 min in M8 medium supplemented with methylcellulose (catalog no. M 0262; Sigma) to detach the adherent bacteria from the food particles (24). The rumen solids from the FL reindeer were treated with 0.1% (wt/vol) methylcellulose, and the solids from the CF, CS1, and CS4 reindeer were treated with 0.18% (wt/vol) methylcellulose. Afterwards, the mixture was homogenized for 30 s (Polytron PT 10 OD; GmbH, Kinematica Luzern, Switzerland) at speed setting 5 and was then serially diluted and inoculated in M8L medium.

Isolation of bacteria. Bacteria were isolated inside the anaerobic chamber as described by Orpin et al. (32, 33). Bacteria from both the rumen fluid and rumen solids from the four FL reindeer were isolated and identified, whereas only bacteria from the rumen fluid from two CF and CS4 reindeer were isolated and identified. Colonies from rumen fluid from the FL and CF reindeer were isolated at a dilution of 10^{-8}, and colonies from rumen solids from the FL reindeer were isolated at a dilution of 10^{-9}. Bacteria from CS4 reindeer were isolated at dilutions of 10^{-5} and 10^{-6}. Bacterial colonies were picked at random from the petri dishes and the tubes used for the viable-cell counts by using sterile glass Pasteur pipettes. They were streaked onto M8L medium in petri dishes in the anaerobic chamber and incubated until pure colonies were obtained. Bacteria from the FL reindeer were then transferred to Hungate tubes containing a slope of M8L medium, and bacteria from the CF and CS4 reindeer were transferred to a slope of solidified M8 medium containing 0.2% (wt/vol) glucose and 0.2% (wt/vol) cellobiose. The tubes were incubated for 24 h and stored at −80°C until they were identified.

Identification of bacteria. The bacteria were identified by standard microbiological techniques (15, 23, 29, 30, 36), including studies of morphology and motility after growth in liquid M8G medium (M8 medium containing 0.2% [wt/vol] glucose for the bacteria from the FL reindeer and 0.5% [wt/vol] glucose for the bacteria from the CF and CS4 reindeer), Gram staining by Hucker's method (30) after growth in liquid M8G medium for 4 h at 39°C, determination of spore formation, analysis of acidic fermentation products, and analysis of substrate utilization patterns. On the basis of these characteristics, genus names were assigned for most of the different isolates, thus placing them in what we consider an appropriate position in the existing classification. Acidic fermentation products were measured after the bacteria were grown in liquid M8G medium for two 24-h periods and 5 ml of the bacterial suspension was fixed in 1.25 ml of 0.5 M HCl. The fermentation products of bacteria from the FL reindeer were determined as described by Sørmo et al. (37). Fermentation products from the bacterial suspensions from the CF and CS4 reindeer were also acidified and extracted with ether and identified by gas liquid chromatography (37a). The extract was injected onto a chromatographic column (Supelco Nukol capillary column; length, 30 m; inner diameter, 0.25 mm) containing 0.25-μm-particle-size silica gel. Succinate and lactate production was determined in a similar way after methylation. The substrate utilization pattern was determined by replica plating from master plates containing eight isolates on M8L medium onto solidified M8 medium (control) containing 2.5% (vol/vol) sheep rumen fluid and onto solidified M8 medium containing 2.5% (vol/vol) sheep rumen fluid and the substrate under examination at a concentration of 0.5% (wt/vol) (32). Growth was detected after 24 to 48 h of incubation at 39°C with reference to the control plate. To detect bacteria fermenting carboxymethyl cellulose (CMC active), the colonies were plated on solidified M8 medium containing 0.2% (wt/vol) cellobiose and 0.1% (wt/vol) low-viscosity CMC (catalog no. C 8758; Sigma). After incubation for 24 h at 39°C, the plates were stained with Congo red (38). Presumptively cellulolytic isolates showed a zone of clearing, unstained by Congo red, around the colony. Aerobic growth was examined on nutrient agar (Difco Laboratories, Detroit, Mich.) containing 0.2% (wt/vol) glucose incubated at 39°C.

Transmission electron microscopy. Fresh particles of the lichen *Cladonia stellaris*, collected from the pasture where the FL reindeer lived, and samples of both fluid and solids from the rumens of the FL reindeer and of rumen fluid from the CF and CS4 reindeer were fixed in 4% glutaraldehyde in 0.1 M Sørensen's phosphate buffer, pH 7.0 (phosphate-buffered saline [PBS]). The samples of rumen fluid and solids were obtained by straining rumen contents through two layers of muslin. Material prepared for transmission electron microscopy (TEM) was postfixed in 1% OsO_4 for 2 h, washed in PBS, stained in 5% uranyl acetate for 1.5 h, and dehydrated in an ethanol series. Fresh and partly digested particles of the lichen *Cladonia stellaris* from the rumen solids were then embedded in Epon-Araldite resin. The rumen fluid was solidified in 10% gelatin in PBS before being embedded in Epon-Araldite resin. The blocks were cut on an RMC MT-7 ultramicrotome, stained with uranyl acetate and Reynolds lead citrate, and observed by TEM (JEOL JEM 1010 microscope). Rumen solid samples from the CF and CS4 reindeer were prefixed for ≥1 h in 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). These samples were then fixed for ≥2 h in 5% glutaraldehyde in 0.1 M cacodylate buffer. Next, the samples were washed twice (for 20 min each time) in 0.1 M cacodylate buffer and then stored in the same buffer at 4°C. Partly digested lichen particles of *Cladonia stellaris* from the rumen solids were prepared for examination as described above.

Scanning electron microscopy. Fresh particles of *Cladonia stellaris*, collected from the pasture, were fixed in 4% glutaraldehyde in 0.1 M PBS. The fixed material was postfixed in 1% OsO_4 for 1.5 h, dehydrated in an ethanol series, and critical point dried in CO_2 before being glued to aluminum stubs with silver glue, sputter coated with gold, and examined in a JEOL JAM 840 scanning electron microscope.

Rumen pH and VFA and lactic acid concentrations. Rumen samples from the four FL reindeer were strained through two layers of muslin, and the pH of the filtrate was measured with a calibrated portable pH meter (PHM 80; Radiometer, Copenhagen, Denmark) with a combined pH electrode (GK 2501C; Radiometer). Duplicate samples (5 ml each) of rumen filtrate were fixed in 1.25 ml of 0.5 M HCl for volatile fatty acid (VFA) analysis as described by Sørmo et al. (37). The VFA and pH values presented from the FL reindeer were extrapolated to zero time as described by Carroll and Hungate (5), Hungate (17), Hungate et al. (18), and White and Staaland (40). The rumen samples from the four CF, CS1, and CS4 reindeer were strained through two layers of muslin, and the pH of the filtrate was measured immediately with the calibrated portable pH meter described above. Duplicate samples (1 ml each) of rumen filtrate were fixed with 3 ml of 60% ethanol and centrifuged (40,000 × *g* for 20 min at 5°C), and the supernatant was collected and stored at −20°C prior to analysis. Samples were thawed and analyzed for VFA and lactic acid concentrations by high-pressure liquid chromatography (HPLC) with a Waters Millipore model 510 HPLC (Waters Millipore, Milford, Mass.) fitted with an HPX 87H column.

Statistical methods. Data on the viable number of bacteria in the rumen fluid and adhering to the rumen solids, ruminal pH, and total VFA and lactic acid concentrations are given as median values and ranges. The viable bacterial populations in the rumen fluid and the VFA concentrations in the rumens of the FL and CF reindeer were compared by the Wilcoxon rank-sum test for comparison of two treatments (22). The null hypothesis was rejected at a P of <0.05.

TABLE 1. Numbers of viable anaerobic bacteria in rumen fluid and rumen solids in reindeer

Reindeer group	Animal	No. of viable anaerobic bacteria (10^8)[a]	
		Per ml of rumen fluid	Per g (wet wt) of rumen solids
FL	A	18.0 (15.0–21.0)	445.0 (400.0–500.0)
	B	24.0 (15.0–25.0)	260.0 (180.0–310.0)
	C	22.0 (17.0–27.0)	450.0 (400.0–500.0)
	D	35.0 (28.0–42.0)	330.0 (250.0–350.0)
CF	E	34.0 (30.0–40.0)	21.0 (15.0–26.0)
	F	26.0 (23.0–29.0)	38.0 (37.0–41.0)
CS1	E	3.3 (2.2–3.5)	15.0 (13.0–19.0)
	F	1.6 (1.3–2.0)	13.0 (12.0–13.0)
CS4	E	0.1 (0.09–0.1)	1.9 (1.8–3.1)
	F	0.09 (0.08–0.1)	0.5 (0.3–0.5)

[a] Data are medians, with ranges in parentheses.

RESULTS

Viable-cell counts. The total viable populations of anaerobic bacteria in the rumen fluid and adhering to the rumen solids in the FL, CF, CS1, and CS4 reindeer are shown in Table 1. In the CF reindeer, the population density in the rumen fluid was similar to that in the FL reindeer ($P > 0.05$). The median population density of bacteria adhering to the rumen solids in the CF reindeer was 92.4% lower than the adherent bacterial population in the FL reindeer. The bacterial population density in the rumen fluid decreased by 91.8 and 99.7% after 1 and 4 days of starvation, respectively. The population density of bacteria adhering to the rumen solids decreased by 52.5 and 95.9% after 1 and 4 days of starvation, respectively.

Identification of bacteria. The bacterial genera isolated from the rumen fluids of the FL, CF, and CS4 reindeer are listed in Table 2. The compositions of the bacterial populations isolated from the FL reindeer differed among the animals. Strains of *Bacteroides* (irregular rods; gram negative; obligately anaerobic; fermentation products are acetate, propionate, and lactate or succinate) dominated in two of the animals (A and B), while strains of *Fibrobacter* (pleomorphic ovoid cells; gram negative; obligately anaerobic; CMC active; fermentation products are acetate, succinate, and propionate) and *Streptococcus* (cocci in chains; gram positive; facultatively anaerobic; fermentation products are lactate and acetate) dominated in animals D and C, respectively. In addition, strains of *Fusobacterium* (irregular rods; gram negative; obligately anaerobic; fermentation products are *n*-butyrate [some strains] as well as acetate, lactate, and succinate), *Butyrivibrio* (curved slim rods; gram negative; obligately anaerobic; motile; fermentation product is *n*-butyrate), and *Clostridium* (straight rods; gram variable; obligately anaerobic; spore forming; fermentation products are acetate, isocaproate, lactate, and *n*-butyrate) were isolated. All of the *Streptococcus* strains were facultatively anaerobic and utilized starch, suggesting that they might be *Streptococcus bovis*.

In the rumen fluids of both CF reindeer, strains of *Streptococcus* and *Clostridium* dominated, while strains of *Eubacterium* (irregular rods; gram positive; obligately anaerobic; fermentation products are acetate and *n*-butyrate) and the family *Enterobacteriaceae* (short straight rods [single and in pairs]; gram negative; facultatively anaerobic; motile; fermentation product is acetate) were represented in small numbers (Table 2). Most of the *Streptococcus* strains (79%) were facultatively anaerobic and utilized starch, suggesting that they might be *S. bovis*. After 4 days of starvation, strains of *Fusobacterium*, the family *Enterobacteriaceae*, and *Eubacterium* dominated (Table 2). In addition, strains of *Bacteroides* and *Streptococcus* were isolated from one of the animals.

The bacteria that adhered to the rumen solids were characterized only for the FL reindeer. Strains of *Bacteroides*, *Fibrobacter*, *Fusobacterium*, *Butyrivibrio*, *Streptococcus*, and *Clostridium* were isolated, but not from all of the animals (Table 3). A *Butyrivibrio* sp. was the only bacterial strain which was isolated from all animals, and it dominated in one (animal B). In animals A, C, and D, strains of *Clostridium*, *Streptococcus*, and *Bacteroides* dominated, respectively. Most of the *Streptococcus* strains (87.5%) were facultatively anaerobic and utilized starch, suggesting that they might be *S. bovis*.

Some bacteria (coccoid and straight rods) were impossible to identify by the standard microbiological techniques (15, 23, 30, 36) and are presented as "Others" in Tables 2 and 3. Biochemical characteristics of the anaerobic bacterial population adhering to the rumen solids of the FL reindeer and the bacterial populations in the rumen fluids of the FL, CF, and CS4 reindeer are presented in Table 4.

Electron microscopy. Ultrathin sections of strained rumen fluid of one FL, one CF, and one CS4 reindeer were examined by TEM (Fig. 1). The micrographs show a diverse bacterial population in all of the animals. The dominant morphological types in the micrographs from the FL and CF reindeer are cocci and straight and coccoid rods. Some of these bacteria

TABLE 2. Proportional composition of the total viable anaerobic bacterial population isolated from reindeer rumen fluid

Type of bacterium	% of total population							
	FL reindeer				CF reindeer		CS4 reindeer	
	A (20)[a]	B (20)	C (20)	D (20)	E (55)	F (35)	E (37)	F (45)
Bacteroides spp.	40.0	65.0	—[b]	15.0	—	—	—	8.9
Fibrobacter spp.	—	10.0	5.0	60.0	—	—	—	—
Fusobacterium spp.	—	10.0	5.0	—	—	—	70.3	28.9
Butyrivibrio sp.	10.0	5.0	—	—	—	—	—	—
Streptococcus spp.	—	—	85.0	20.0	67.3	42.9	—	2.2
Clostridium spp.	35.0	5.0	—	5.0	23.7	17.1	—	—
Eubacterium spp.	—	—	—	—	1.8	17.1	2.7	22.2
Enterobacteriaceae	—	—	—	—	1.8	5.8	16.2	37.8
Others	15.0	5.0	5.0	—	5.4	17.1	10.8	—

[a] Numbers in parentheses are numbers of strains isolated from the rumen fluid of each reindeer.
[b] —, not detected.

TABLE 3. Proportional composition of the total viable anaerobic bacterial population isolated from FL reindeer rumen solids

Type of bacterium	% of total population in reindeer			
	A (20)[a]	B (20)	C (17)	D (20)
Bacteroides spp.	—[b]	15.0	11.8	75.0
Fibrobacter spp.	—	10.0	—	10.0
Fusobacterium spp.	—	5.0	—	5.0
Butyrivibrio sp.	40.0	30.0	23.5	5.0
Streptococcus spp.	5.0	5.0	35.3	—
Clostridium spp.	55.0	20.0	—	5.0
Others	—	15.0	29.4	—

[a] Numbers in parentheses are numbers of strains isolated from the rumen solids of each reindeer.
[b] —, not detected.

looked quite similar to the isolated bacteria such as *Streptococcus* spp., *Bacteroides* spp., and *Clostridium* spp. In the micrograph from a starved animal, cocci are observed together with a few slightly curved rods, short straight rods, and small irregular bacteria resembling *Butyrivibrio fibrisolvens*, members of the *Enterobacteriaceae*, and *Fusobacterium* spp., respectively.

A photograph and a scanning electron micrograph of a fresh particle of the lichen *Cladonia stellaris* are shown in Fig. 2A and B, respectively. These panels show the outside layer of the lichen, which is composed of fungus hyphae and algae. Ultrathin cross sections of fungus hyphae of *Cladonia stellaris*, fresh (Fig. 2C) and partly digested in the rumen of an FL (Fig. 2D), a CF (Fig. 2E), and a CS4 (Fig. 2F) reindeer were examined by TEM. The bacteria seem to attack and digest the lichen fungus hyphae from the lumen rather than from the surface. In the micrographs of partly digested lichen particles from all of the animals, the bacteria resembling *Bacteroides* spp. have extensively penetrated the lichen cell wall. In addition to the *Bacteroides* spp., the dominant morphological bacterial types in the FL reindeer are straight rods resembling *Fusobacterium* spp. or *Clostridium* spp. which contain large glycogen deposits. In the CF reindeer, coccus-shaped bacteria dominated, and a few straight and slightly curved rods were seen. These resembled *Fusobacterium* spp. or *Clostridium* spp. and *B. fibrisolvens*. In the starved reindeer, coccus-shaped bacteria dominated, and a few small irregular types were seen.

Rumen pH and VFA and lactic acid concentrations. The median ruminal pH, total VFA and lactic acid concentrations, and acetate/propionate/butyrate ratios in the rumen fluids of FL, CF, CS1, and CS4 reindeer are shown in Table 5. The total VFA concentration was significantly higher in the FL reindeer ($P < 0.03$) than in the CF reindeer. The acetate/propionate ratios in the CF and FL reindeer were 3.0 and 4.0, respectively.

DISCUSSION

The density of viable bacteria in the rumen fluid of both the FL and the CF reindeer was only about one-fifth of the density of bacteria found in the rumen fluid of domestic ruminants eating alfalfa hay and a concentrate (mainly consisting of corn and soya bean meal) in a 1/2 ratio (25). The low bacterial density in the rumen fluid of the CF reindeer may be explained by a relatively low availability of water-soluble carbohydrates (2.0% of DM) (12) in the lichen *Cladonia stellaris*. Data on the chemical composition of reindeer forage in northern Norway in winter are limited but indicate that the main carbohydrates of the plants that reindeer eat are plant cell wall polysaccharides that are highly lignified and not easy for the rumen bacteria to digest (26). This could explain the low population density of bacteria in the rumen fluid of even the FL reindeer in comparison with domestic ruminants eating alfalfa hay and concentrates (25).

The bacterial population density in the rumen fluid decreased by 99.7%, from 10^9 to 10^6 cells per ml, during 4 days of starvation (Table 1). The rumen fluid turnover time does not change during 4 days of starvation in reindeer that are fed lichens before starvation (1). This contributes to a higher rate of depletion of bacteria from the rumen fluid in reindeer than in sheep, in which the rumen turnover time is decreased during starvation (19).

In the CF reindeer, the densities of bacteria adherent to rumen solids were similar to the population densities of bacteria in the rumen fluid and were about 8% of the densities of adherent bacteria in the FL reindeer. The pure lichen (*Cladonia stellaris*) contained 3.1% of its DM as crude protein (20), and the plant fraction of the rumen content from the FL reindeer contained 9.6% of its DM as crude protein (26). The higher protein content in the mixed winter diet than in the pure lichen diet could stimulate bacterial growth and explain

TABLE 4. Biochemical characteristics of the anaerobic bacterial population isolated from rumen solids and rumen fluid of four FL reindeer, two CF reindeer, and two CS4 reindeer

Characteristic	% of total population with characteristic			
	FL reindeer		CF reindeer rumen fluid (90)	CS4 reindeer rumen fluid (82)
	Rumen solids (77)[a]	Rumen fluid (80)		
Facultatively anaerobic	13.0	26.3	74.4	26.8
Gram positive or variable	35.1	43.8	94.4	20.7
Motile	32.5	1.3	25.6	30.5
Utilization of:				
Glucose	100	100	88.4	81.7
Fructose	100	100	NM[b]	NM
Maltose	NM	NM	84.4	81.7
Cellobiose	68.8	67.5	NM	NM
Starch	96.1	71.3	82.2	85.4
Xylan	37.7	22.5	4.4	0
Lichenin	5.2	12.5	0	0
Pectin	26.0	15.0	0	0
CMC	29.9	32.5	0	0

[a] Numbers in parentheses are numbers of strains isolated from each group of reindeer.
[b] NM, not measured.

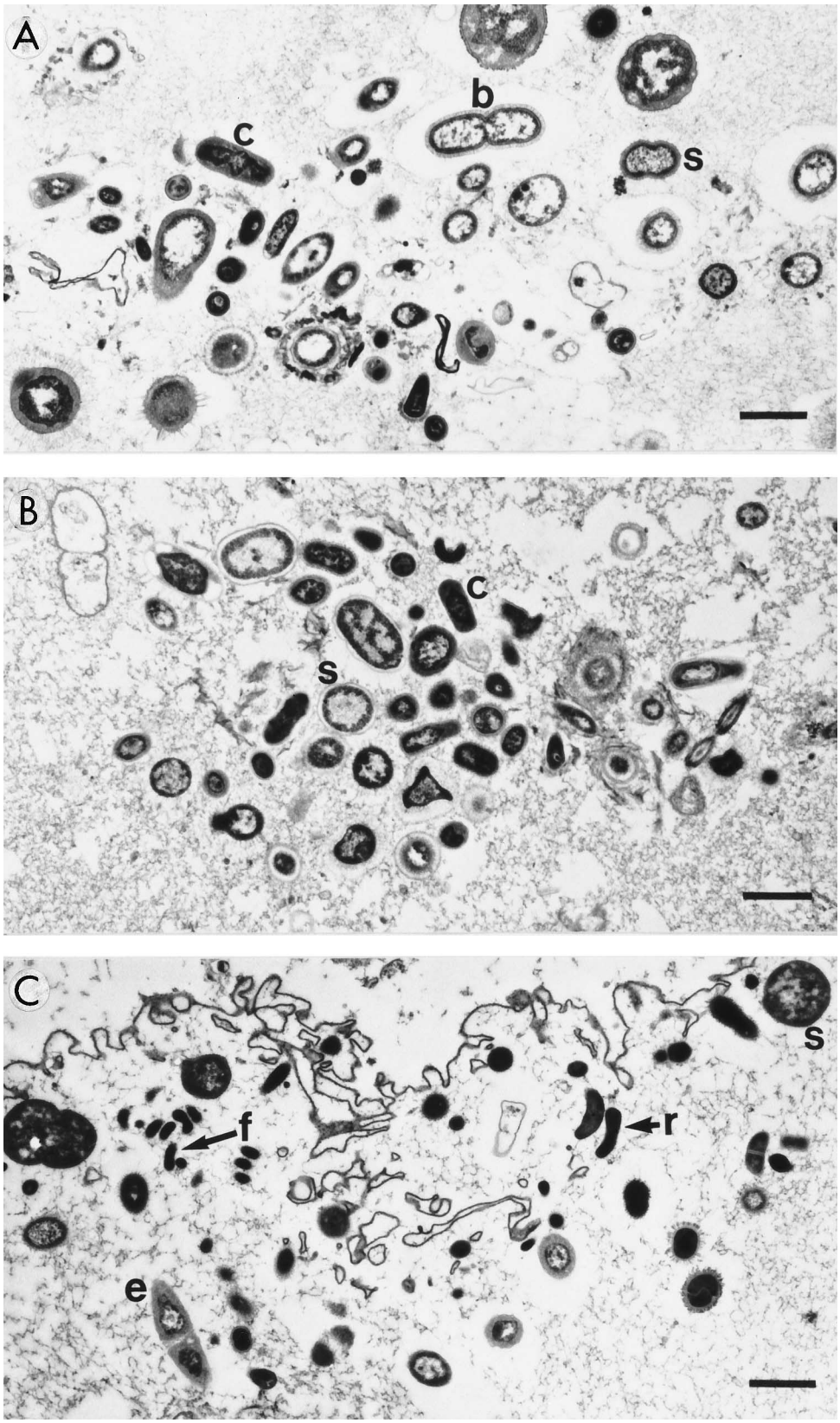

FIG. 1. Transmission electron micrographs of strained rumen fluid from an FL reindeer (A), a CF reindeer (B), and a CS4 reindeer (C). In the samples from the FL and the CF reindeer, coccoid bacteria resembling *Streptococcus* spp. (s) and straight rods resembling *Clostridium* spp. (c) are shown. Bacteria resembling *Bacteroides* spp. (b) are shown only in the micrograph of the sample from the FL reindeer. In the sample from the CS4 reindeer, a bacterium resembling a *Streptococcus* sp. (s) is shown, as are different rod-shaped bacteria resembling *Fusobacterium* spp. (f), *B. fibrisolvens* (r), and bacteria of the family *Enterobacteriaceae* (e). Bars = 1 μm.

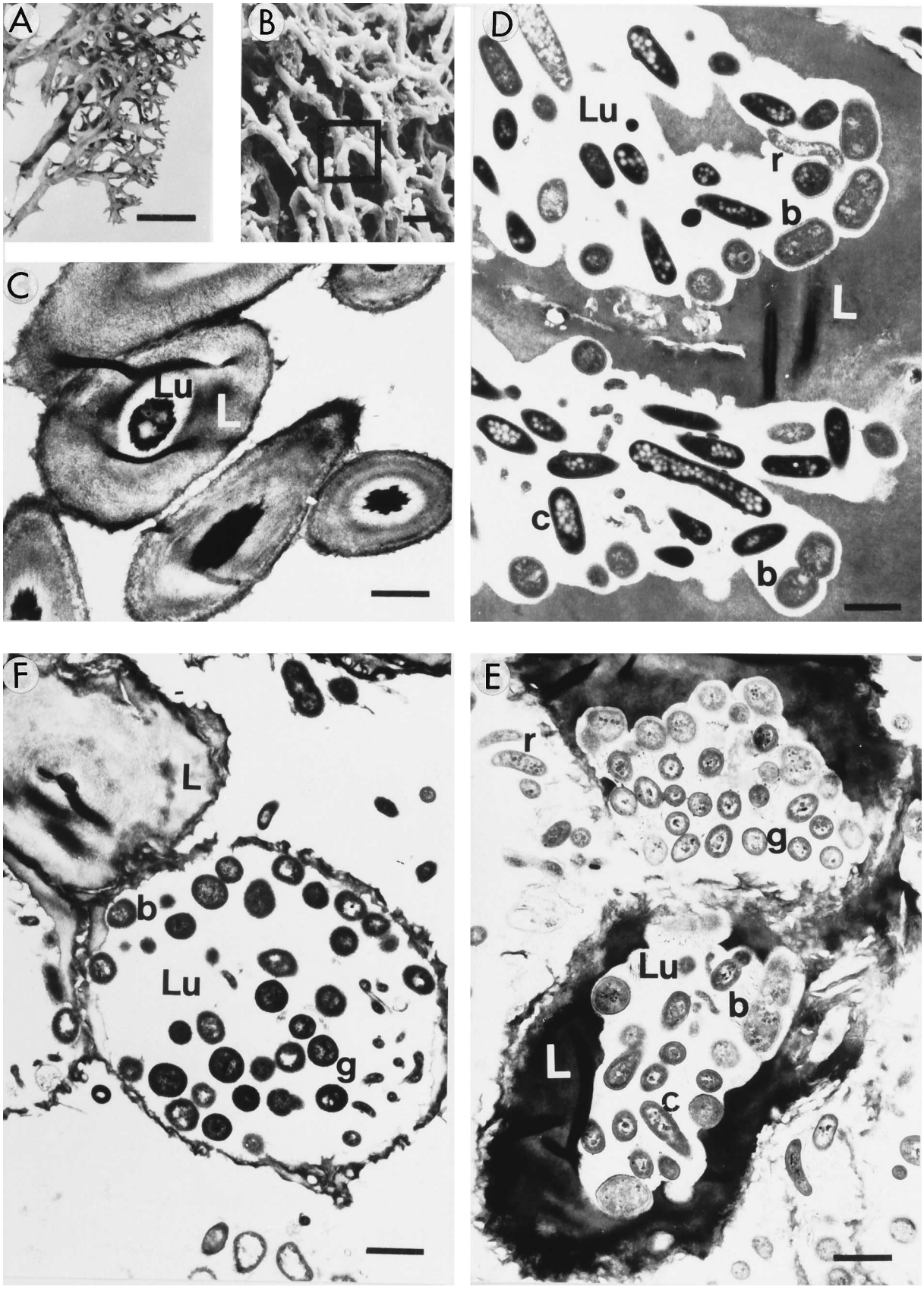
A
B
C
Lu
L
D
Lu
r
b
L
c
b
F
L
b
Lu
g
E
r
g
Lu
b
L
c

FIG. 2. (A) Photograph of a fresh *Cladonia stellaris* lichen particle. (B) Scanning electron micrograph of a small section of the outside layer of a fresh lichen particle composed of fungus hyphae and algae. (C) Transmission electron micrograph representing a magnification of a cross section of fungus hyphae (area outlined in panel B). L indicates the fungus hyphae; the lumen of the hyphae is indicated by Lu. Also shown are transmission electron micrographs of cross sections of partly digested *Cladonia stellaris* particles taken from the rumen of an FL reindeer (D), a CF reindeer (E), and a CS4 reindeer (F). The adherent cells in panels D to F resemble *Bacteroides* spp. (b). Note the large number of cells containing large glycogen deposits in panel D. Bacterial cells resembling *Fusobacterium* spp. or *Clostridium* spp. (c) are shown in panels D and E. Curved rods (r) resembling *B. fibrisolvens* are in close association with the adherent population in panels D and E. Coccoid bacteria (g) are shown in large numbers in panels E and F. It seems that bacteria attack and digest the lichen from the lumen but not from the surface. Bars: 0.5 cm (A), 10 μm (B), and 1 μm (C to F).

the high number of bacteria that adhered to the rumen solids in the FL reindeer. The bacterial population adhering to the rumen solids decreased at a lower rate during starvation than the bacterial population in the rumen fluid (Table 1). Rumen solids have a longer retention time in the rumen than rumen fluid has, and the ability of the bacteria to adhere to the plant particles (7) could explain this finding.

Considering the unusual nature of lichens as food for ruminants, it was surprising to find that the bacterial populations isolated from the rumens of the FL, CF, and CS4 reindeer were almost the same as those known to occur in domestic ruminants (17), although those from the CF and CS4 reindeer showed major differences in the dominant species (Table 2). The important difference between the rumen bacterial populations in the rumen fluid of the FL reindeer and those in the CF reindeer was the presence of *Bacteroides* spp. and *Fibrobacter* spp. in three of the FL animals. The isolation of *Bacteroides* spp. and *Fibrobacter* spp. from the FL reindeer but not from the CF reindeer could be due to the high cellulose content in the diet of the FL reindeer (26). In one of the FL reindeer, *Streptococcus* was the dominant bacterial genus. The individual differences in the compositions of the ruminal bacteria of animals from the same pasture that were killed at the same time of day are difficult to explain, but the individual animals could have been exposed to different gradations of starvation during the winter.

In the CF reindeer, *Streptococcus* was the dominant bacterial genus in the rumen fluid. *S. bovis* usually ferments starch and would have access to some of the carbohydrates in the lichens. *S. bovis* was dominant in the rumen fluid of sheep that ate seaweed (32), which, like lichens, is low in cellulose and which has a carbohydrate fraction consisting of poorly digested glycoprotein polymers and polysaccharides containing unusual sugars (6, 32). Of the bacterial strains isolated from the rumen fluid and adhering to the rumen solids of the FL reindeer, 32.5 and 29.9%, respectively, were CMC active. In the CF and CS4 reindeer, none of the bacteria isolated from the rumen fluid was CMC active. This suggests that prolonged feeding on lichens influences the cellulolytic population of bacteria in reindeer. The lichen *Cladonia stellaris* contains only 1.7% of its DM as cellulose, but 78.4% of its DM is hemicellulose (34). Considering the high hemicellulose content, it was surprising that bacterial strains such as *Fibrobacter*, *Butyrivibrio*, and *Ruminococcus* strains, which usually hydrolyze hemicellulose, were not isolated from the rumen fluid of the CF reindeer. It is known that in domestic ruminants, hemicellulose from vascular plants is resistant to degradation in the rumen and becomes available for microbial fermentation in the lower gut after being treated by acid and enzymes in the abomasum and the small intestine (39). The pattern of hemicellulose fermentation in the reindeer rumen is not understood.

Lichenin, which was used in the culture medium and was originally isolated from the lichen *Cetraria islandica*, was utilized by 12.5 and 5.2% of the isolated bacteria from the rumen fluid and those adhering to the rumen solids, respectively, in FL reindeer. The lack of bacteria that fermented lichenin in the rumen fluid in the CF and CS4 reindeer was probably due to a low lichenin content in *Cladonia stellaris*. This finding indicates that a natural mixed winter diet dominated by a mixture of different lichens and supplemented by woody plants, grasses, and mosses stimulates growth of both CMC-active and lichenin-fermenting bacteria in the rumen fluid.

The composition of the bacterial population in the rumen fluid changed considerably after 4 days of starvation, when strains of *Fusobacterium*, the family *Enterobacteriaceae*, and *Eubacterium* dominated (Table 2). This pattern reflected major changes in the rumen, including a decrease in both DM content, which decreased from 14 to 3% (1), and pH, which increased from 6.6 to 7.6 (Table 5). Strains of *Fusobacterium* and *Eubacterium* grow fastest at a pH near 7.0. Members of the *Enterobacteriaceae* are normally not common in the rumen.

Transmission electron micrographs of the rumen fluids of one FL, one CF, and one CS4 reindeer revealed some bacteria with morphologies identical to those of the isolated bacteria. It is difficult to characterize the bacterial populations in rumen fluid with transmission electron micrographs, but some obvious differences between the animals were seen. For example, small, irregular bacteria resembling strains of *Fusobacterium* were apparent only in the micrograph of rumen fluid from the starved reindeer.

Transmission electron micrographs of partly digested lichen particles taken from the rumens of one FL, one CF, and one

TABLE 5. Reindeer ruminal pHs, total VFA and lactic acid concentrations, and acetate/propionate/butyrate ratios[a]

Reindeer group[b]	pH	Concn (mmol/liter) of:		Acetate/propionate/butyrate ratio
		VFAs	Lactic acid	
FL	6.5 (6.3–6.7)	78.9 (70.4–82.6)	NM[c]	72:18:11, (69–74):(15–22):(7–11)
CF	6.6 (6.5–6.6)	66.7 (57.4–72.1)	1.6 (0–3.2)	69:23:8, (62–70):(21–31):(7–9)
CS1	7.2 (7.1–7.2)	37.2 (30.4–40.5)	0.3 (0–0.7)	81:19:7, (73–82):(5–20):(0–13)
CS4	7.6 (7.6–7.8)	19.9 (13.7–37.2)	ND[d]	85:16:0, (68–100):(0–32):(0)

[a] Data are medians, with ranges in parentheses or following commas.
[b] Four reindeer per group.
[c] NM, not measured.
[d] ND, not detected.

CS4 reindeer showed that bacteria resembling *Bacteroides* spp. degrade the lichen particles. The micrographs of the partly digested lichen particles (Fig. 2D to F) show that the bacteria attack and digest the lichen from the lumen of the fungus hyphae but not from the surface. Rumen bacteria penetrate the grasses via the cuticle through stomata or breaks caused by prehension or chewing and reach the underlying cells of the epidermis, mesophyll, bundle sheaths, and phloem, which they digest (2, 14, 28). *Cladonia stellaris* has no cuticle or epidermis, and the fungus hyphae are therefore easily attacked by the bacteria.

The VFA concentrations in the rumens of the FL reindeer were higher than those in the rumens of the CF reindeer ($P < 0.03$), indicating a high rate of fermentation and better growth conditions for bacteria in the FL reindeer. The ratio of acetate to propionate indicates a higher level of ruminal fermentation of plant cell wall fibers in the FL reindeer than in the CF reindeer (Table 5). The presence of fiber-digesting bacteria, which produce acetate as a major end product, could contribute to the ruminal acetate/propionate ratio in the FL reindeer. The low acetate/propionate ratio of 3.0 in the CF reindeer was unexpected, since only 2.0% of the DM is made up of water-soluble carbohydrates and 78.4% is made up of hemicellulose in *Cladonia stellaris* (12, 34). Lichens are considered to be an unusual diet for ruminants, and it is therefore possible that other components of the lichens influence the VFAs produced. During starvation, profound effects on the ruminal VFA concentration, pH, and DM content were observed and may influence both the bacterial number and composition.

The results of this study indicate that microbial digestion in the reindeer rumen in winter is stimulated by intake of a mixed diet and not only by lichens. The rumen bacteria mostly attack and digest the lichen hyphae from the inside. The lichens are composed mainly of structural carbohydrates, such as hemicellulose, and give the rumen bacteria a source of energy but provide suboptimal growth conditions because of their low protein content. A mixed winter diet increases the animals' protein intake, which could improve the growth conditions for the rumen bacteria. When the reindeer are exposed to acute starvation, the ruminal microbial environment changes considerably.

ACKNOWLEDGMENTS

We thank Johan Mathis M. Sara and Mathis Sara for providing the FL reindeer. Colin G. Orpin is acknowledged for helpful discussion during the planning of this project. We thank Randi Olsen for preparing samples for TEM.

This work was supported by the Reindeer Research Fund under the auspices of the Norwegian Ministry of Agriculture.

REFERENCES

1. **Aagnes, T. H., and S. D. Mathiesen.** 1994. Food and snow intake, body mass and rumen function in reindeer fed lichen and subsequently starved for 4 days. Rangifer **14:**33–37.
2. **Akin, D. E., and H. E. Amos.** 1975. Rumen bacterial degradation of forage cell walls investigated by electron microscopy. Appl. Microbiol. **29:**692–701.
3. **Bøe, U.-B., and E. Jacobsen.** 1981. Trials with different feeds to reindeer. Rangifer **1:**39–43.
4. **Bryant, M. P., and I. M. Robinson.** 1961. An improved nonselective culture medium for ruminal bacteria and its use in determining diurnal variation in numbers of bacteria in the rumen. J. Dairy Sci. **44:**1446–1456.
5. **Carroll, E. J., and R. E. Hungate.** 1954. The magnitude of the microbial fermentation in the bovine rumen. Appl. Microbiol. **2:**205–214.
6. **Cheeke, P. R.** 1991. Applied animal nutrition, feeds and feeding. Macmillian Publishing Company, New York.
7. **Costerton, J. W., G. G. Geesey, and K.-J. Cheng.** 1978. How bacteria stick. Sci. Am. **238:**86–95.
8. **Culberson, C. F.** 1969. Chemical and botanical guide to lichen products. The University of North Carolina Press, Chapel Hill.
9. **Dehority, B. A.** 1975. Characterization studies of rumen bacteria isolated from Alaskan reindeer (*Rangifer tarandus* L.), p. 228–240. *In* J. R. Luick, P. C. Lent, D. R. Klein, and R. G. White (ed.), Proceedings of the First International Reindeer and Caribou Symposium. University of Alaska, Fairbanks.
10. **Dehority, B. A., and J. A. Grubb.** 1976. Basal medium for the selective enumeration of rumen bacteria utilizing specific energy sources. Appl. Environ. Microbiol. **32:**703–710.
11. **Dougherty, R. W.** 1955. Permanent stomach and intestinal fistulas in ruminants: some modifications and simplifications. Cornell Vet. **45:**331–357.
12. **Drake, B.** 1943. Untersuchungen über einige Polysaccharide der Flechten, vornehmlich das Lichenin und das neuentdeckte Pustulin. Biochem. Z. **313:** 388–399.
13. **Hale, M. E.** 1961. Lichen handbook. Smithsonian Institution Press, Washington, D.C.
14. **Harbers, L. H.** 1985. Ultrastructural utilization of plants by herbivores. Food Microstruct. **4:**357–364.
15. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore.** 1973. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
16. **Hungate, R. E.** 1950. The anaerobic mesophilic cellulolytic bacteria. Bacteriol. Rev. **14:**1–49.
17. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press, Inc., New York.
18. **Hungate, R. E., R. A. Mah, and M. Simesen.** 1961. Rates of production of individual volatile fatty acids in the rumen of lactating cows. Appl. Microbiol. **9:**554–561.
19. **Hyden, S.** 1961. Determination of the amount of fluid in the reticula-rumen of sheep and its rate of passage to the omasum. K. Lantbrukshögsk. Ann. **27:**51–79.
20. **Jacobsen, E., and S. Skjenneberg.** 1975. Some results from feeding experiments with reindeer, p. 95–107. *In* J. R. Luick, P. C. Lent, D. R. Klein, and R. G. White (ed.), Proceedings of the First International Reindeer and Caribou Symposium. University of Alaska, Fairbanks.
21. **Jarett, I. G.** 1948. The production of rumen and abomasal fistulae in sheep. J. Counc. Sci. Ind. Res. **21:**311–315.
22. **Johnson, R. A., and G. K. Bhattacharyya.** 1992. Statistics: principles and methods, 2nd ed. John Wiley & Sons, Inc., New York.
23. **Krieg, N. R., and J. G. Holt (ed.).** 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
24. **Kudo, H., K.-J. Cheng, and J. W. Costerton.** 1987. Electron microscopic study of the methylcellulose-mediated detachment of cellulolytic rumen bacteria from cellulose fibers. Can. J. Microbiol. **33:**267–272.
25. **Leedle, J. A. Z., M. P. Bryant, and R. B. Hespell.** 1982. Diurnal variations in bacterial numbers and fluid parameters in ruminal contents of animals fed low- or high-forage diets. Appl. Environ. Microbiol. **44:**402–412.
26. **Mathiesen, S. D., et al.** Unpublished data.
27. **Mathiesen, S. D., A. Rognmo, and A. S. Blix.** 1984. A test of the usefulness of a commercially available mill "waste product" (AB-84) as feed for starving reindeer. Rangifer **4:**28–34.
28. **Monson, W. G., J. G. Powell, and G. W. Burton.** 1972. Digestion of fresh forage in the rumen fluid. Agron. J. **64:**231–233.
29. **Montgomery, L., B. Flesher, and D. Stahl.** 1988. Transfer of *Bacteroides succinogenes* (Hungate) to *Fibrobacter* gen. nov. as *Fibrobacter succinogenes* comb. nov. and description of *Fibrobacter intestinalis* sp. nov. Int. J. Syst. Bacteriol. **38:**430–435.
30. **Ogimoto, K., and S. Imai.** 1981. Atlas of rumen microbiology. Japan Scientific Societies Press, Tokyo.
31. **Olsen, M. A., T. H. Aagnes, and S. D. Mathiesen.** 1994. Digestion of herring by indigenous bacteria in the minke whale forestomach. Appl. Environ. Microbiol. **60:**4445–4455.
32. **Orpin, C. G., Y. Greenwood, F. J. Hall, and I. W. Paterson.** 1985. The rumen microbiology of seaweed digestion in Orkney sheep. J. Appl. Bacteriol. **59:**585–596.
33. **Orpin, C. G., S. D. Mathiesen, Y. Greenwood, and A. S. Blix.** 1985. Seasonal changes in the ruminal microflora of the high-Arctic Svalbard reindeer (*Rangifer tarandus platyrhynchus*). Appl. Environ. Microbiol. **50:**144–151.
34. **Person, S. J., R. G. White, and J. R. Luick.** 1980. Determination of nutritive value of reindeer-caribou range, p. 224–239. *In* E. Reimers, E. Gaare, and S. Skjenneberg (ed.), Proceedings of the 2nd International Reindeer and Caribou Symposium. Direktoratet for vilt og ferskvannsfisk, Trondheim, Norway.
35. **Skogland, T.** 1975. Range use and food selectivity by wild reindeer in southern Norway, p. 342–352. *In* J. R. Luick, P. C. Lent, D. R. Klein, and R. G. White (ed.), Proceedings of the First International Reindeer and Caribou Symposium. University of Alaska, Fairbanks.
36. **Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.).** 1986. Bergey's manual of systematic bacteriology, vol. 2. Williams & Wilkins, Baltimore.

37. **Sørmo, W., T. H. Aagnes, M. A. Olsen, and S. D. Mathiesen.** The bacteriology of the small intestinal mucosa of free-living reindeer. Rangifer **14:**65–78.
37a. **Supelco, Inc.** 1985. GC bulletin 748H. Supelco, Inc., Bellefonte, Pa.
38. **Teather, R. M., and P. J. Wood.** 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. **43:**777–780.
39. **Van Soest, P. J.** 1982. Nutritional ecology of the ruminant. Cornell University Press, Ithaca, N.Y.
40. **White, R. G., and H. Staaland.** 1983. Ruminal volatile fatty acid production as an indicator of forage quality in Svalbard reindeer. Acta. Zool. Fenn. **175:**61–63.